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Complement-Dependent Lysis of Influenza A Virus-Infected Cells by Broadly Cross-Reactive Human Monoclonal Antibodies[▽]

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We characterized human monoclonal antibodies (MAbs) cloned from influenza virus-infected patients and from influenza vaccine recipients by complement-dependent lysis (CDL) assay. Most MAbs active in CDL were neutralizing, but not all neutralizing MAbs can mediate CDL. Two of the three stalk-specific neutralizing MAbs tested were able to mediate CDL and were more cross-reactive to temporally distant H1N1 strains than the conventional hemagglutination-inhibiting and neutralizing MAbs. One of the stalk-specific MAbs was subtype cross-reactive to H1 and H2 hemagglutinins, suggesting a role for stalk-specific antibodies in protection against influenza illness, especially by a novel viral subtype which can cause pandemics.

Human influenza is a highly contagious acute respiratory illness that is responsible for significant morbidity and excess mortality, especially in the elderly and the very young worldwide. Every year in the United States, on average 5% to 20% of the population acquires influenza, more than 200,000 people are hospitalized for influenza complications, and influenza-related deaths range from 3,000 to 49,000. The elderly, young children, and individuals with certain health conditions are at high risk for serious influenza complications (Centers for Disease Control and Prevention [http://www.cdc.gov/flu/about/disease/index.htm]). Current vaccine approaches depend primarily on the induction of antibodies to the viral surface protein hemagglutinin (HA). Serum hemagglutination inhibition (HAI) titers to the circulating virus of 1:40 or greater are associated with significant protection against influenza illness (15). In the elderly, however, HAI titers measured pre- and postvaccination were not distinguishable between subjects who subsequently developed influenza illness and those who did not (12), showing the limitation of the HAI titer as an indicator of protection in this population.

Antibodies inducing HAI and neutralization are generally considered subtype specific and bind to the globular head region of the HA, a receptor binding site (14). In 1993, however, a mouse monoclonal antibody (MAb), C179, which neutralizes H1, H2, H5, and H9 subtypes, was isolated (13, 18; C179 datasheet [http://catalog.takara-bio.co.jp/en/PDFFiles/M145_DS_e.pdf]). Recently, four groups reported human MAbs with similar characteristics which were able to neutralize group 1 (H1, H2, H5, H6, H8, H9, H11, H12, H13, and H16 based on phylogenetic analysis [17]) influenza A viruses (1, 2, 20). These stalk region-specific antibodies cannot inhibit hemagglutination (2, 13, 20, 23). The presence of these MAbs indicates that at the clonal level, some neutralizing and hemagglutination-inhibiting antibodies are distinct and their activities are not correlated.

In addition to the neutralization of cell-free virus by antibodies to HA and the interference of virus release from infected cells by antibodies to neuraminidase (NA), influenza virus-specific antibodies bind to infected cells and are able to lyse the virus-infected cells through activation of complement (complement-dependent lysis [CDL]) (16, 21). The complement system plays several roles in response to influenza virus infection. In primary infection with influenza virus, mice deficient in component C3 showed delayed viral clearance and increased viral titers in lungs (9). The addition of complement can enhance the neutralization of influenza virus by antibodies *in vitro* (5). Complement is also known to enhance influenza virus-specific $CD4^+$ and $CD8^+$ T cell responses and to help maintain long-term memory of influenza viruses in mice (3, 9). Complement, therefore, can link innate and adaptive immunities and is probably important to consider for vaccine development (4).

In this study, we analyzed 13 HA-specific human MAbs molecularly cloned from plasmablasts obtained from patients infected with 2009 pandemic influenza (23) or from recipients of prepandemic seasonal influenza vaccines (24) by CDL assay, which is a modification of a method reported previously (16, 21). Cells from the human lung cancer cell line A549 (type II alveolar epithelial cells) (11) infected with influenza virus were used as targets instead of mouse kidney or embryo cells. All MAbs have the same constant region of human IgG1 subclass (the variable region of an antibody was cloned by reverse transcription [RT]-PCR and recombined with the constant region of IgG1), the most abundant subclass which can activate the classical pathway of the complement system (7, 8). These MAbs were categorized into four different groups based on

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TABLE 1. CDL activities of MAbs against target cells infected with 2009 pandemic or seasonal H1N1 influenza A virus strains

MAb	Virus specificity[a]	% specific lysis of cells infected with indicated viruses[b]	
		Pandemic H1N1	Seasonal H1N1
MN^+/HAI^+ (globular head specific)			
1009-3B06	Pandemic and seasonal H1N1	**17.8**	**15.7**
1009-3F01	Pandemic and seasonal H1N1	**14.1**	−8.6
EM-4C04	Pandemic H1N1	−3.4	−7.3
TIV-1[c]	Seasonal H1N1	−4.6	**23.6**
MN^+/HAI^- (stalk specific)			
1009-3B05	Pandemic and seasonal H1N1 and H5N1[d]	**13.7**	**43.8**
1009-3E06	Pandemic and seasonal H1N1 and H5N1[d]	−2.1	**28.1**
70-1F02	Pandemic and seasonal H1N1 and H5N1[d]	**14.2**	**39.9**
MN^-[e]$/HAI^+$			
TIV-2	Seasonal H1N1	−4.9	**19.6**
MN^-/HAI^-			
1000-1B02	Pandemic and seasonal H1N1[f]	−4.8	−3.4
1000-2C02	Pandemic and seasonal H1N1[f]	−5.6	−3.7
70-1D02	Pandemic and seasonal H1N1[f]	−5.3	−7.5
70-5C05	Pandemic and seasonal H1N1[f]	−3.1	−2.0
70-5D01	Pandemic and seasonal H1N1[f]	0.2	−3.1

[a] Data are from reference 23 except those for MAbs TIV-1 and TIV-2.
[b] MAbs were tested at 10 μg/ml in CDL assays. Low-Tox guinea pig complement (Cedarlane Laboratories, Burlington, NC) was added at a final dilution of 1:20. Target cells are A549 cells infected with seasonal [A/Solomon Islands/3/2006 (H1N1)] or 2009 pandemic [A/California/7/2009 (H1N1)] influenza A virus strains at multiplicities of infection of 5 to 10. Percent specific lysis is calculated as (% lysis by antibody + complement) − (% lysis by complement only)/(% maximum lysis) − (% lysis by complement only). Maximum lysis was obtained by lysing target cells by Renex 30 detergent (Uniqema, New Castle, DE). All assays were performed in triplicate. Positive lysis results are shown in bold.
[c] Not experimentally determined to bind to the globular head.
[d] Bound to H5 HA in ELISA.
[e] Not neutralizing at the highest concentration tested (30 μg/ml).
[f] Bound to 2009 pandemic and seasonal H1 HAs in ELISA.

their microneutralization (MN) and HAI titer patterns against 2009 pandemic [A/California/4/2009 (H1N1)] or seasonal (A/Solomon Islands/3/2006) H1N1 strains (Table 1).

First we tested these 13 MAbs for CDL activity against target cells infected with 2009 pandemic or seasonal H1N1 strains at a concentration of 10 μg/ml (Table 1). Six MAbs (1009-3B06, TIV-1, 1009-3B05, 1009-3E06, 70-1F02, and TIV-2) showed moderate to high levels of specific immune lysis (16 to 44%) by CDL of seasonal H1N1 influenza virus-infected target cells, and four MAbs (1009-3B06, 1009-3F01, 1009-3B05, and 70-1F02) showed moderate levels (14 to 18%) of CDL activity on 2009 pandemic H1N1 virus-infected target cells (in CDL assays, approximately 40 to 60% of target cells were infected with influenza virus; results were confirmed by fluorescence-activated cell staining [data not shown]). Five MAbs which showed binding only to whole viruses by enzyme-linked immunosorbent assay (ELISA; MN^-/HAI^- MAbs) did not have CDL activity at 10 μg/ml. Subsequently, the seven MAbs which mediated CDL at 10 μg/ml were tested at lower concentrations (Fig. 1). These MAbs retained CDL activity at concentrations as low as 0.04 to 4 μg/ml. There were three MAbs which mediated CDL against both 2009 pandemic H1N1-infected and seasonal H1N1 virus-infected target cells (1009-3B06, 1009-3B05, and 70-1F02). For these MAbs, higher concentrations were needed to mediate CDL against the 2009 pandemic H1N1 virus-infected target cells than for those infected with the seasonal A/Solomon Islands/3/2006 (H1N1) strain, which is consistent with previous analyses in that MAbs 1009-3B06 and 70-1F02 had higher minimum effective concentrations against the 2009 pandemic virus than the seasonal viruses in MN assays (23). At these minimum effective concentrations for MN, both MAbs were also active in CDL assays (Fig. 1). MAb TIV-1, specific to seasonal H1N1, was active only in a CDL assay against seasonal H1N1 strain-infected target cells.

MN and CDL antibody assay results, however, did not always correlate. Seasonal H1N1-specific MAb TIV-2, which was not neutralizing at 30 μg/ml, mediated CDL at as low as 4 μg/ml. MAbs 1009-3E06 and EM-4C04, which were neutralizing at concentrations similar to those for MAbs 1009-3B06, 1009-3F01, 1009-3B05, and 70-1F02, did not mediate CDL.

Others have reported that stalk-specific neutralizing MAbs were more cross-reactive than conventional globular head-specific neutralizing MAbs (1, 2, 13, 20). Therefore, we tested these three stalk-specific MAbs (all are encoded by the *VH1-69* gene [23]) as well as three other MAbs (1009-3B06, TIV-1, and TIV-2) which showed CDL activity only against target cells infected with recent seasonal H1N1 virus strains (Table 1 and Fig. 1) in CDL assays against target cells infected with temporally distant seasonal H1N1 strains (isolated from 1934 to 2007) (Fig. 2). We found that the three stalk-specific MAbs lysed target cells infected with all the H1N1 strains tested. In contrast, the other three MAbs lysed only target cells infected with recent seasonal H1N1 strains (Fig. 2). One of the three stalk-specific MAbs (70-1F02) lysed target cells infected with an H2N1 reassortant virus, X-27 (Fig. 3A). The percent specific lysis values for MAb 70-1F02 against the H2N1-infected cells were significantly higher than those for MAb 1009-3B05 at both concentrations tested (Fig. 3B).

Our results showed that most MAbs active in CDL were neutralizing, but not all neutralizing MAbs can mediate CDL of target cells. Two of the three stalk-specific neutralizing MAbs and three of the four conventional globular head-specific MN^+/HAI^+ MAbs and one MN^-/HAI^+ MAb were able to mediate CDL. Among the MAbs active in CDL, the stalk-specific MAbs were more cross-reactive than the globular head-specific MAbs. One potential explanation for the stalk-specific MAbs being more active in CDL may be the relative proximity of their binding sites on the HA to the infected cell membrane (2, 13, 20), on which the activated proteins in the complement system are deposited, resulting in lysis of the cell (7). However, we do not know which subclasses the MAbs we have tested in CDL assays originally belonged to, because we did not clone the constant regions of the MAbs. About 60% of human IgG is IgG1, but these MAbs can belong to the IgG3 subclass, a stronger activator of the complement system, or to the IgG2 or IgG4 subclass, both of which are weaker or are not activators (8). One of the three stalk-specific MAbs, 70-1F02,

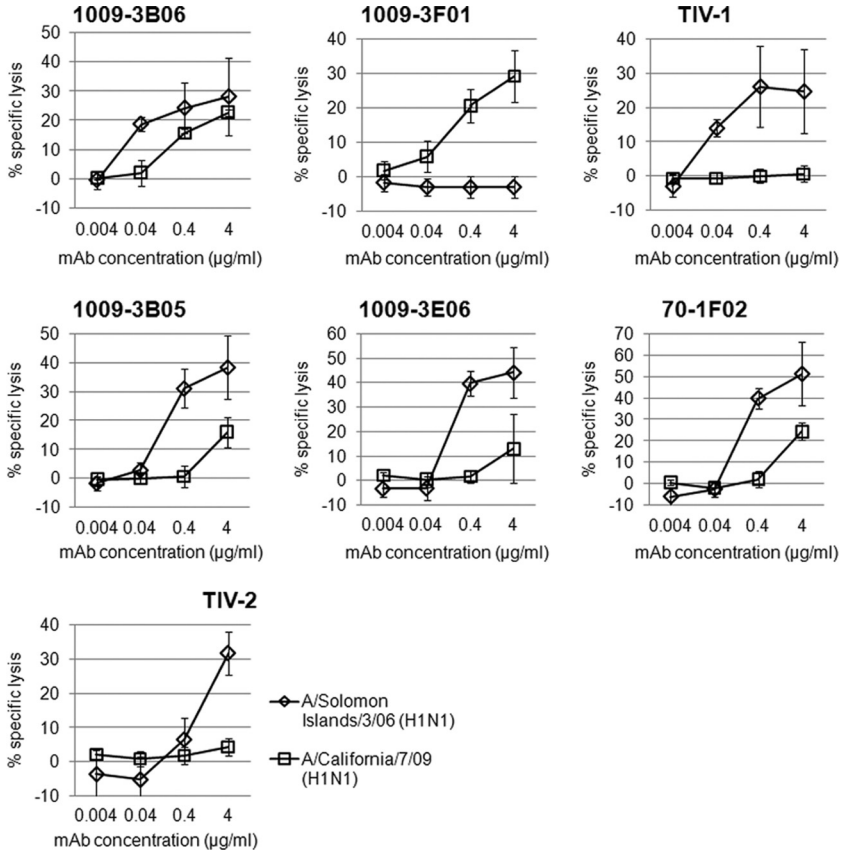

FIG. 1. CDL activities against target cells infected with seasonal H1N1 and the 2009 pandemic H1N1 strains by seven MAbs at lower antibody concentrations. MAbs 1009-3B06, 1009-3F01, TIV-1, 1009-3B05, 1009-3E06, 70-1F02, and TIV-2, which had shown moderate to high levels (14 to ~44%) of CDL lysis of A/Solomon Islands/3/2006 (H1N1)- and/or A/California/7/2009 (H1N1)-infected A549 target cells (Table 1), were tested at lower antibody concentrations against target cells infected with the same seasonal H1N1 and 2009 pandemic H1N1 strains. All assays were performed in triplicate. Averages and standard deviations for three independent experiments are shown.

was subtype cross-reactive to H2 HA. It is likely that this MAb recognizes an epitope slightly different from those of other stalk-specific MAbs or binds to the same epitope at a different angle.

Recently, Steel et al. reported the generation of new influenza vaccines (a combination of a DNA vaccine and a virus-like particle) based on the stalk region and their immunogenicity in mice (19). Wei et al. reported induction of stalk-

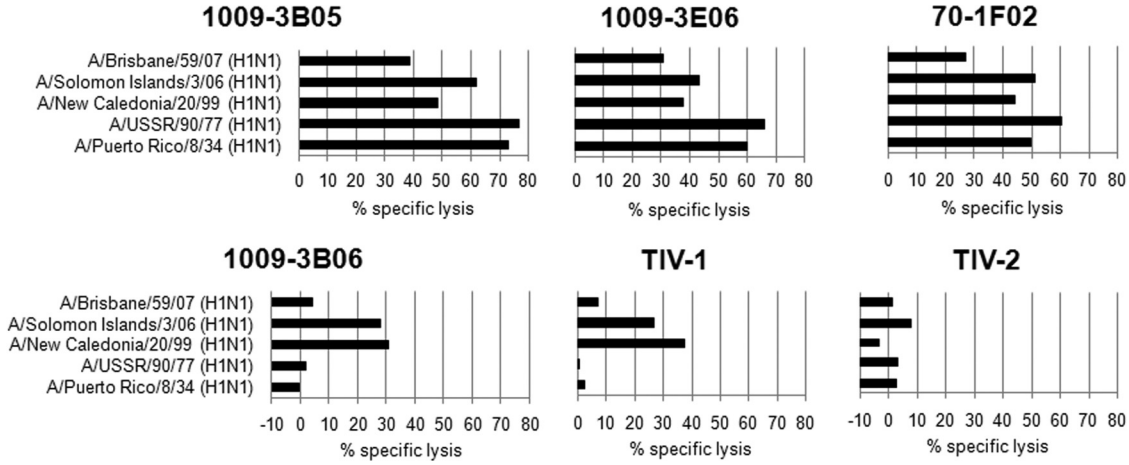

FIG. 2. Cross-reactivities of MAbs against target cells infected with temporally distant seasonal H1N1 strains. Six MAbs were tested at 4 μg/ml (MAbs 1009-3B06 and TIV-2 were tested at 5 μg/ml) against A549 cells infected with recent seasonal strains A/Brisbane/59/2007 (H1N1), A/Solomon Islands/3/2006 (H1N1), and A/New Caledonia/20/1999 (H1N1) and older strains A/USSR/90/1977 (H1N1) and A/Puerto Rico/8/1934 (H1N1) in CDL assays. Low-Tox guinea pig complement was added at a final dilution of 1:20. All assays were performed in triplicate.

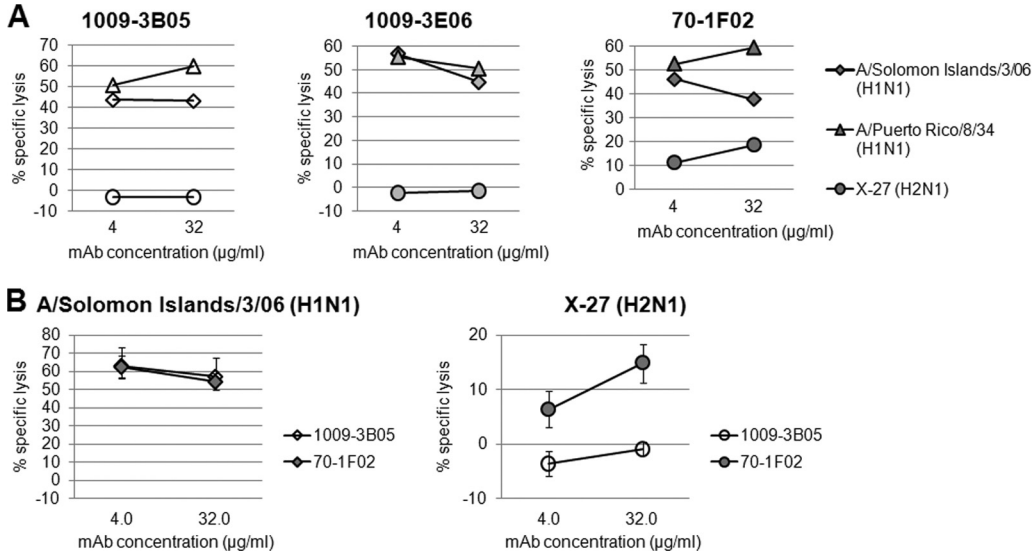

FIG. 3. Subtype cross-reactivity of MAb 70-1F02 against target cells infected with H1N1 and H2N1 strains. (A) CDL assays were used to test the stalk-specific MAbs 1009-3B05 (white), 1009-3E06 (light gray), and 70-1F02 (dark gray) against target cells infected with influenza virus strains A/Solomon Islands/3/2006 (H1N1) and A/Puerto Rico/8/1934 (H1N1) and a reassortant virus, X-27 (H2N1), which has the HA of A/Rockefeller Institute/5/1957 (H2N2) and the NA of A/NWS/1934 (H1N1). All assays were performed in triplicate. (B) Two MAbs, 1009-3B05 and 70-1F02, were compared in CDL assays against target cells infected with the A/Solomon Islands/3/2006 (H1N1) virus or the X-27 (H2N1) reassortant virus. All assays were performed in triplicate. Averages and standard deviations for three independent experiments are shown. The percent specific lysis values for MAb 70-1F02 were significantly higher against the H2N1-infected cells than those for MAb 1009-3B05 at both 4 and 32 μg/ml ($P = 0.013$ and $P = 0.018$, respectively, as determined by Student's t test, assuming unequal variances).

specific antibodies by a prime/boost combination of plasmid DNA plus an adenovirus vector or plasmid DNA plus a seasonal vaccine in mice, ferrets, and monkeys (22). Corti et al. cloned memory B cells producing subtype cross-reactive stalk-specific IgG from four healthy human donors who received a seasonal influenza vaccine (1), while Wrammert et al. cloned human MAbs only from 2009 pandemic influenza virus-infected patients, but not from seasonal influenza vaccine recipients (23). It would be helpful to learn how common these subtype-cross-reactive stalk-specific antibodies are and about their biologic functions *in vivo*.

Although primary infection of BALB/c mice induced CDL antibodies that were subtype specific (21), human studies conducted after the reemergence of the H1N1 subtype in 1977 found that sera from three young adults, who were unlikely to have previously experienced H1N1 influenza viruses (they were exposed to H2N2 and H3N2 subtypes between 1957 and 1976, but not to the H1N1 subtype), showed low levels of CDL against target cells infected with A/USSR/90/1977 (H1N1) (16). These low levels of CDL to "novel" influenza virus subtypes may be mediated by antibodies cross-reactive to H1 and H2 HAs. A study using mouse MAbs showed that, in addition to anti-HA antibodies, NA- and nucleoprotein-specific MAbs mediated CDL of influenza virus-infected target cells, but matrix protein 1-specific MAbs did not (25). Recently Grandea et al. reported that human MAbs specific to an epitope on the highly conserved ectodomain of matrix protein 2 (M2) were able to mediate CDL of CHO cells stably expressing M2 (6). M2 is known to be expressed abundantly at the cell surface (10). Therefore, it is also possible that antibodies to NA, nucleoprotein, and M2 may also contribute to CDL subtype cross-reactivity but probably to a lesser degree.

In conclusion, we have shown that antibodies that bind to the stalk region of the HA molecule can not only neutralize free influenza virus particles but can also eliminate virus-infected cells through CDL. These CDL antibodies are broadly cross-reactive within an HA subtype, and some appear to be subtype cross-reactive, suggesting their role in protection against influenza illness, especially in heterologous protection.

Nucleotide sequence accession numbers. Variable region sequences of all MAbs except for TIV-1 and -2 have been deposited in GenBank under accession numbers HQ689787, HQ689752, HQ689762, HQ689763, HQ689731, HQ689727, HQ689750, HQ689751, HQ689789, HQ689761, HQ689778, HQ689776, HQ689713, HQ689705, HQ689702, HQ689718, HQ689774, HQ689785, HQ689783, HQ689766, HQ689772, and HQ689756.

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